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Transgenic restorer rice line T1c-19 with stacked *cry1C*/bar* genes has low weediness potential without selection pressure



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Abstract

Stacked (insect and herbicide resistant) transgenic rice T1c-19 with *cry1C*/bar* genes, its receptor rice Minghui 63 (herein MH63) and a local two-line hybrid *indica* rice Fengliangyou Xiang 1 (used as a control) were compared for agronomic performance under field conditions without the relevant selection pressures. Agronomic traits (plant height, tiller number, and aboveground dry biomass), reproductive ability (pollen viability, panicle length, and filled grain number of main panicles, seed set, and grain yield), and weediness characteristics (seed shattering, seed overwintering ability, and volunteer seedling recruitment) were used to assess the potential weediness without selection pressure of stacked transgene rice T1c-19. In wet direct-seeded and transplanted rice fields, T1c-19 and its receptor MH63 performed similarly regarding vegetative growth and reproductive ability, but both of them were significantly inferior to the control. T1c-19 did not display weed characteristics; it had weak overwintering ability, low seed shattering and failed to establish volunteers. Exogenous insect and herbicide resistance genes did not confer competitive advantage to transgenic rice T1c-19 grown in the field without the relevant selection pressures.

Keywords: stacked transgenic rice, T1c-19, agronomic traits, reproductive ability, weediness

1. Introduction

Globally biotech crops reached 181.5 million hectares in 2014, greatly contributing to higher productivity and providing economic benefits to farmers (James 2014). Compared to mono-trait crops, gene-stacked transgenic crops offer durable multi-toxin resistance to target pests or resistance

to herbicides, making them suitable for complex farming conditions. Stacked genes have become commercially very important and a future trend for transgene technology; 51 million hectares equivalent to 28% of the total area planted with transgenic crops corresponded to crops with stacked traits in 2014 (James 2014).

Rice, the most important food crop, was among of the first ones to which biotechnology was applied to develop herbicide-resistant, high-yielding, high-quality, stress-tolerant varieties (Huang *et al.* 2002; Jia and Peng 2002; Rong *et al.* 2006). In 2009, Ministry of Agriculture of China issued a security certificate for the production of the insect-resistant, transgenic rice Huahui 1 (a restorer line) and hybrid rice *Bt* Shanyou 63, both stacked with the *cryAb1/cryAc1* genes, representing a major step towards the commercialization of transgenic rice in China. Among transgenic rice developed in China, T1c-19 was obtained by transforming Minghui 63,

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an elite CMS-restorer line used in the production of several widely grown rice hybrids, with both the insect resistance *cry1C** gene and the glufosinate resistance *bar* gene (Tang *et al.* 2006), making it useful for developing combined insect and herbicide-resistant hybrids.

Biosafety evaluation is important for transgenic crop research and a requirement for their commercialization. Key issues of the environmental risk assessment (ERA) of transgenic crops include: transgene escape to weedy or wild relatives through hybridization and introgression; their potential weediness through volunteerism or de-domestication; effects on non-target wild species and biodiversity, and the evolution of resistance in the target organisms or to the chemical (in the case of herbicides) being used to control weeds (Conner *et al.* 2003; Andow and Zwahlen 2006; Wolt 2009). The first two are the major avenues for a transgenic crop becoming a weed. Regarding transgenic rice, risk of transgene movement into conspecific weedy rice (*Oryza sativa* L.) is of major concern. Previous studies have clearly demonstrated that transgenic rice hybridizes with weedy rice (Chen *et al.* 2004; Messeguer *et al.* 2004; Zhang *et al.* 2006; Olguin *et al.* 2009; Chun *et al.* 2011; Zuo *et al.* 2011; Sun *et al.* 2015). Moreover, fitness of hybrids between transgenic rice and weedy rice increased compared to non-transgenic plants or the weedy parent under certain conditions (Xia *et al.* 2011; Yang *et al.* 2011, 2012). Therefore gene flow from transgenic rice to weedy rice should not be ignored. The gene flow from T1c-19 to weedy rice and its cultivated rice receptor Minghui 63 have been evaluated (Huang *et al.* 2015).

The potential weediness of transgenic crops themselves is also an important consideration for the successful commercialization of transgenic crops. The possession of modified genes with strong selective advantage traits (including insect or herbicide resistance and environmental stress tolerance) may increase the fitness associated to competitiveness and invasiveness of transgenic crops under selection pressure (Eastick and Hearnden 2009). Therefore it is important to clearly demonstrate that the transgenic crop is no more likely to generate a weed problem than its non-transgenic counterpart (Purrington and Bergelson 1995).

Characteristics associated with weediness include discontinuous germination and long-lived seeds; rapid seedling growth; rapid growth to reproduction stage, especially under unfavorable condition, very high seed output or vigorous vegetative reproduction; adaptations for dispersal; and competitiveness (De Wet and Harlan 1975; Muenscher 1980). Although these characteristics have been bred out of most crops during domestication, crops vary in their degree of domestication (Warwick and Stewart 2005; Roberts *et al.*

2014). Consequently the environmental risk assessment should consider whether the transgenic plant is similar to the non-transgenic counterpart with respect to traits identified as being important for survival and persistence in the environment (Roberts *et al.* 2014). When transgenic crops perform differently from non-transgenic crops in an agricultural setting, these differences might be reflected in their relative invasiveness in natural habitats. Therefore, differences between transgenic and non-transgenic lines should be quantified on vegetative growth, reproductive ability and seed characteristics (Purrington and Bergelson 1995; Roberts *et al.* 2014).

Crawley *et al.* (2001) monitored four transgenic crops (oilseed rape, potato, maize and sugar beet) over 10 years in 12 different habitats, and in no case were the genetically modified plants found to be more invasive or more persistent than their conventional counterparts. Eastick and Hearnden (2009) examined the potential weediness of *Bt* cotton in northern Australia, and found that there was no difference between the transgenic line and the non-transgenic counterpart in germination, survival, fecundity and invasiveness, indicating that the addition of the *Bt* gene did not confer increased fitness for weediness. Song *et al.* (2009) reported that the survival and competitive ability of transgenic glyphosate-resistant soybean 40-3-2 did not pose a potential for weediness. Similarity, single or multiple herbicide-resistant oilseed rape (*Brassica napus*) was not more weedy than non-herbicide resistant plants in ruderal sites and natural areas (Simard *et al.* 2005), although herbicide-resistant transgenic seedlings of oilseed rape were found at several ports, roadsides and harbors in Japan (Saji *et al.* 2005; Kawata *et al.* 2009), Canada (Yoshimura *et al.* 2006; Knispel *et al.* 2008; Knispel and McLachlan 2010) and in U.S (Schafer *et al.* 2011; Munier *et al.* 2012).

Cui *et al.* (2012) demonstrated that potential weediness of glufosinate-resistant rice Minghui 86B was low, because of its lower survival and reproductive ability, seed viability than its conventional counterpart rice Minghui 86 and cultivated hybrid Shanyou 63. However, compared to mono-trait crop varieties, stacked transgenic plants may be more prone to become weedy due to the additional advantage traits that could confer advantage under certain environment conditions. Huang *et al.* (2014) assessed the potential weediness of stacked transgene rice B2A68 (*Cry2Aa#* and *bar*) in the field, and found that the vegetative growth, fecundity, and seed viability of B2A68 were similar to those of Bar68-1 with *bar* gene. However, a transgenic rice line containing the *CpTI* gene alone was similar in fecundity relative to non-transgenic controls, but under insect pressure, *Bt/CpTI* rice lines had 47% greater fecundity than

the non-transgenic controls (Yang *et al.* 2011). Similarly, double transgenic tobacco lines (glyoxalase I+glyoxalase II) always withstood salinity stress better than either of the single gene-transformed lines and wild type plants under salinity stress (Singla-Pareek *et al.* 2003).

Resistance performance (Tang *et al.* 2006; Zhang *et al.* 2011), effect on biodiversity (Lu *et al.* 2014), and food safety assessment (Cao *et al.* 2012) of T1c-19 have been already characterized. This stacked transgenic rice displays a high level of resistance to its target insect leaf folders and stemborers. T1c-19 did not significantly affect non-target organisms and arthropod communities compared to its parental line. T1c-19 has potential to gain regulatory approval. Furthermore, gene flow from T1c-19 to weedy rice was evaluated in 2014 (Huang *et al.* 2015). Hence, our field study concentrated on the weediness of stacked transgene rice T1c-19. The results derived from this study should be useful to regulatory authorities in their risk assessment and decision making related to authorizing the release of this stacked transgenic rice.

2. Results

2.1. Weed occurrence

Relative coverage ratio (RCR) at the first stage reached approximately 60 and 84% at 30 and 50 day after sowing (DAS), respectively (Fig. 1). The dominant weeds were barnyardgrass (*Echinochloa crusgalli* (L.) Beauv.) and flatsedge (*Cyperus iria* L.), which represented 31 and 46% of total weeds RCR at 30 DAS, respectively. Flatsedge became the dominant weed at 50 DAS, making 90% of total weeds RCR. Other species, including Indian toothcup (*Rotala indica* (Willd.) Koehne), Duckweed (*Lemna minor* L.), and Lesser fimbristylis (*Fimbristylis miliacea* (L.) Vahl) were present at lower densities.

At the second stage, RCR decreased significantly to approximately 5% at 30 DAS, with barnyardgrass and flatsedge remaining as the main weeds; however, the RCR of total weeds raised to 90% at 50 DAS without changes in dominance. At the third stage, RCR was 39 and 75% at 30

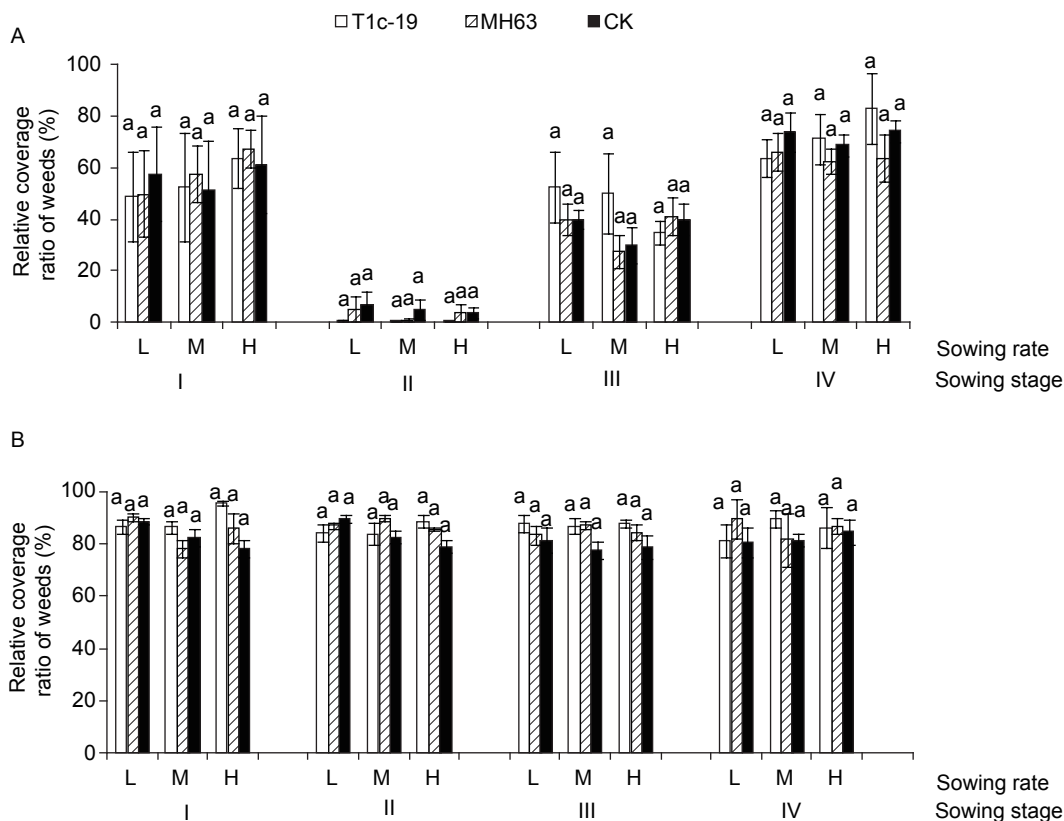


Fig. 1 Relative coverage ratio of weeds at 30 day (A) and 50 day (B) after sowing in wet direct seeded field. MH63, Minghui 63; CK, control cultivar (Fengliangyou Xiang 1); L, M and H represent low, medium and high sowing rates, respectively; I, II, III and IV mean planting on 25th May, 15th June, 5th July and 25th July, respectively. Data are means \pm SE. Columns within sowing rate at the same sowing stage followed by the same letters do not differ according to a Duncan's multiple range test ($P < 0.05$). The same as below.

and 50 DAS, respectively. Asian sprangletop (*Leptochloa chinensis* (L.) Nees) was the main weed, accounting for 50 and 95% of total weeds RCR at 30 and 50 DAS, respectively. After 50 DAS, Asian sprangletop was 20% taller than the experimental rice plants. At the fourth stage, RCR was 75 and 90% at 30 and 50 DAS, respectively, the two most important species being Asian sprangletop and Indian toothcup, the height of former and the latter was 50% taller and 90% shorter than the experimental rice plants, respectively. Regardless of stage, RCR for total weeds was similar across plots in the same sowing rate.

In transplanted plots, flatsedge and barnyard grass were found occasionally. Weed species and RCR were similar across plots.

2.2. Seedling establishment rates

At the first stage, more than 90% of the T1c-19, 68% of the receptor rice MH63 and 80% of the control established at the three sowing rates, respectively. In most cases, the number of plants of the three lines established in the field was similar at each sowing rate. However, at high sowing rate, the transgenic rice had significantly greater establishment rate than the receptor rice MH63. At the second, third and fourth stages, establishment rates of the three rice lines were similar within sowing rate, above 62, 85 and 92%, respectively (Table 1).

2.3. Agronomic traits

Plant height At the first stage, plant height of transgenic

and receptor rice were similar from 30 DAS to maturity regardless of sowing rate. Compared to control, transgenic rice T1c-19 and receptor rice lines were shorter or similar in height at 30, 50 and 70 DAS at the three sowing rates. But at maturity, the transgenic rice and MH63 were more than 16 or 10 cm and 21 or 24 cm shorter than control at low and medium sowing rate, respectively. At high sowing rate, the three rice lines had similar plant height (data not shown). At the second, third and fourth stage, in most cases, transgenic rice and receptor rice were similar on plant height from 30 DAS to maturity at the same sowing rate. The transgenic rice line in all cases and the receptor rice line in most cases were shorter than control at 50, 70 DAS and maturity (data not shown).

In general, the transgenic rice and receptor rice lines were similar in height but significantly shorter than the control at the same time after sowing within sowing rate (Fig. 2).

In the transplanted field, the transgenic and the receptor rice lines were similar in plant height, but shorter approximately 14, 24, 26, 27 cm than control, which were 53.6, 91.5, 105.8, and 120.4 cm at 30, 50, 70 DAS and maturity, respectively.

Tillering Tiller number of the three rice lines within sowing rate was similar (0.2–1.2 and 0.6–2.5 tillers per plant at 30 and 50 DAS, respectively) at the first stage. At 70 DAS, tiller number of the three rice lines decreased to 0.4–1.4 per plant and at maturity, most plants had no tillers at all. At the second stage, from 30 to 70 DAS, in majority cases, the tiller number of the three rice lines within sowing rates was similar (1.8–3.2, 2.1–4.9 and 0.9–3.6 tillers per plant at 30, 50 and 70 DAS, respectively). At maturity, the transgenic

Table 1 Seedling establishment rates of the three rice lines at different sowing rates

Seeding rate (seeds m ⁻²)	Rice line ¹⁾	Wet direct-seeded date (2013)			
		25th May	15th June	5th July	25th July
75	T1c-19	98.83±0.09 a	74.00±0.09 a	90.67±0.15 a	99.78±0.04 a
	MH63	92.67±0.07 a	75.33±0.01 a	99.33±0.13 a	99.30±0.10 a
	CK	87.33±0.07 a	89.17±0.19 a	97.33±0.24 a	92.35±0.08 a
150	T1c-19	91.67±0.12 a	87.33±0.13 a	99.33±0.16 a	99.33±0.09 a
	MH63	80.67±0.12 a	69.33±0.01 a	93.00±0.09 a	98.60±0.11 a
	CK	80.76±0.07 a	62.33±0.08 a	99.67±0.02 a	98.56±0.09 a
300	T1c-19	98.17±0.03 a	62.63±0.09 a	94.00±0.13 a	99.75±0.15 a
	MH63	68.17±0.02 b	67.67±0.08 a	84.83±0.09 a	99.63±0.04 a
	CK	87.67±0.05 a	63.83±0.13 a	87.67±0.09 a	98.89±0.15 a
Mean	T1c-19	98.22	74.72	94.67	99.62
	MH63	80.50	70.78	92.38	99.17
	CK	85.22	71.78	91.56	96.60

¹⁾ MH 63, Minghui 63; CK, control cultivar (Fengliangyou Xiang 1).

The numbers are written by means±SE. Mean values within a column within sowing rate followed by the same letter are not significantly different according to a Duncan's multiple range test ($P < 0.05$).

The same as below.

rice had 1.4, 0.6 and no tillers under low, medium and high sowing rate, which was similar to the receptor rice and control (Fig. 3). At the third stage, tiller number of three rice lines was similar, 0.6–1.4, 0.5–1.2, 0.1–0.6 at three sowing rate from 30 DAS to maturity. At the fourth stage, at 30 and 50 DAS, most plants of the three rice lines had no tillers; a few had 1–2 tillers. At 70 DAS and maturity, all plants of the three rice lines had no tillers (data not shown).

When transplanted, tiller number of the three rice lines

was similar to that at 30 DAS (5.7–7.0 per plant). However, T1c-19 and MH63 had 0.9 or 2, 1.1 or 1.2, 2.9 or 4 more tillers than control at 50, 70 DAS and maturity, respectively. **Aboveground dry biomass** T1c-19 and its receptor MH63 have similar aboveground dry biomass from the first to fourth stages at the same sowing rate. However, T1c-19 and its receptor weighed 33.6–57.6, 23.5–35.7, and 29.1–35.4 g less than that of control in the first, second, and third stages, respectively. In the fourth stage, at low and medium sowing

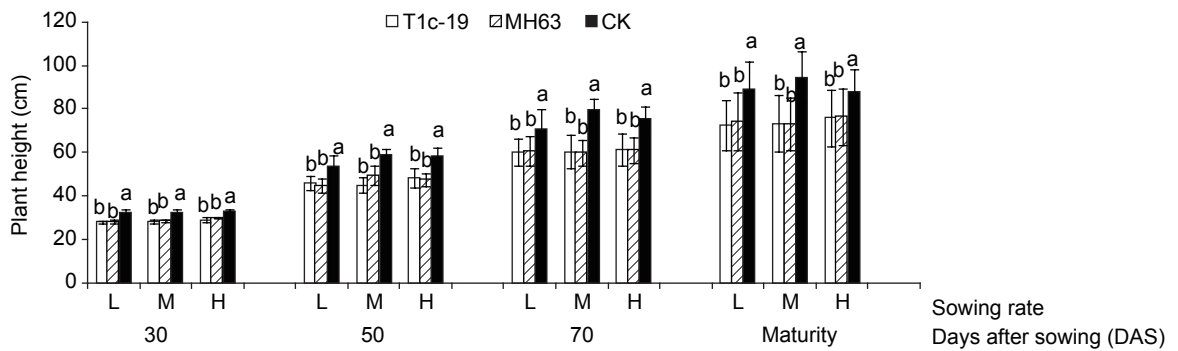


Fig. 2 Plant height of the three rice lines at different sowing rates in a wet direct-seeded field. Data of the same rice line at the same sowing rate from the first to the fourth sowing stage were pooled for analysis.

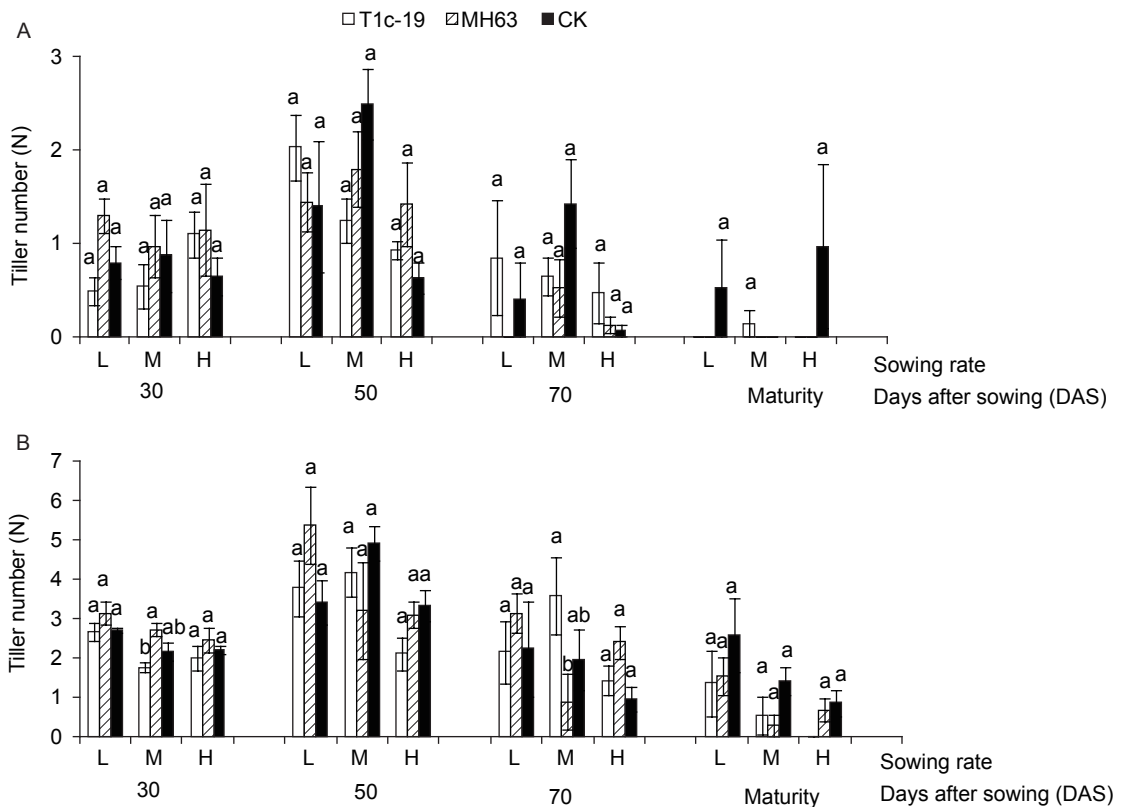


Fig. 3 Tiller number of the three rice lines at different sowing rates in wet direct-seeded field.

rate, both of T1c-19 and its receptor MH63 were significantly lower than control on this value. However at high sowing rate, the aboveground dry biomass of the three rice lines was similar (Fig. 4).

In the transplanted field, the three rice lines had similar aboveground dry biomass, averaging between 58.7 and 72.6 g.

2.4. Reproductive ability

Flowering periods In the wet direct-seeded field, the control first began flowering, reached its blooming peak and completed flowering, followed by both T1c-19 and its receptor rice line MH63 that started flowering 8, 5 and 10 days later than control at the first to third stage. At the fourth stage, T1c-19 and MH63 failed to flower and produce heads,

but control began flowering at 68 DAS (Table 2).

In the transplanted field, T1c-19 and its receptor MH63 also flowered at the same time. Compared to the control, both of them began flowering 6 days later, reached their blooming peak 10 days later and completed flowering 14 days later.

Pollen viability At the three stages, the average pollen viabilities of T1c-19, MH63 and control were similar ($P>0.05$), at 99%. At the fourth stage, pollen viability of the control was over 98%, but T1c-19 and MH63 failed to complete the transition from vegetative to reproductive stage.

Reproductive variables In the wet direct-seeded field, T1c-19 did not differ from its receptor MH63 in panicle length, filled grain number/panicles, seed set, and yield m^{-2} under the same sowing rate at any of the first three stages (Table 3). Compared to the control, panicle length of T1c-19 and MH63 were in most cases shorter at the first three

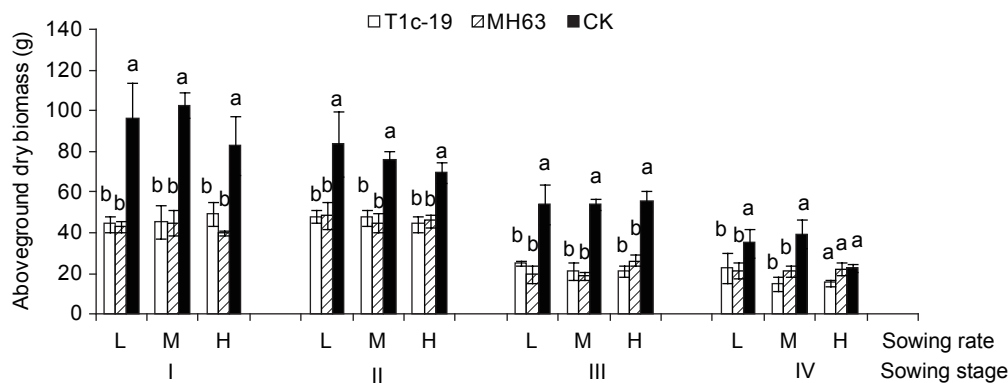


Fig. 4 Aboveground dry biomass of three rice lines at maturity in wet direct-seeded field.

Table 2 Flowering times of three rice lines in direct-seeded and transplanted fields

Planting method	Sowing or transplanting date	Rice line	Beginning flowering date (d)	Peak flowering date (d)	Final flowering date (d)
Direct seeding	25th May	T1c-19	86	94	96
		MH63	86	94	97
		CK	78	82	85
	15th June	T1c-19	76	84	89
		MH63	76	83	89
		CK	71	75	80
	5th July	T1c-19	82	90	95
		MH63	82	90	95
		CK	72	75	79
	25th July	T1c-19	–	–	–
		MH63	–	–	–
		CK	68	70	77
Transplanting	25th June	T1c-19	65	74	80
		MH63	65	74	80
		CK	59	64	66

– means not flowering.

stages, but similar at the fourth stage. Seed set of T1c-19 and MH63 were greater in the first three stages, but lower in the fourth stage. Both of T1c-19 and MH63 had 61–124 less filled grain number/main panicles at three sowing rates in three stages. T1c-19 and MH63 had 0.31–1.19, 1.79–3.42 and 1.88–5.93 kg less yield m^{-2} under low, medium and high sowing rates in three stages.

In the transplanted field (Fig. 4), T1c-19 and MH63 had similar panicle length, filled grain number/panicle, and yield m^{-2} . However, seed set of T1c-19 was approximately 78% significantly lower than that (83%) of MH63. Compared to the control, both of T1c-19 and MH63 had shorter panicles and less filled grains per panicle, seed set, and yield m^{-2} (Table 4).

2.5. Weediness characteristics

Shattering rate Regardless sowing rate and stage, shat-

tering rate of T1c-19 and MH63 were approximately 2.5% and 1.5%, respectively. Both of them shattered significantly less seed than the control at 13.3%.

Volunteer seedling No volunteer seedlings were found during the experimental period.

Seed survival over winter in the field Seed viability similarly decreased over winter among the three rice lines, from 100% to less than 32 and 38% in two months of burial at 3 and 20 cm depth, respectively (Fig. 5). In four months, seed viability of the three rice lines decreased to less than 21.5 and 22.3% at 3 and 20 cm depth, respectively. After six months, seed had lost their viability at both depths, with less than 8 and 8.3% of maximum seed viability at 3 and 20 cm depth. Most non-germinating seeds were non-viable according to the 2,3,5-triphenyltetrazolium chloride (TTC) test, only a very small proportion (2–7% at 3 cm depth and 4–6% at 20 cm depth) were dormant.

Table 3 The reproductive variables of three rice lines in wet direct-seeded field

SD	SR	RL	PL (cm)	FG	SS (%)	Y (kg m^{-2})
25th May	L	T1c-19	24.16±0.76 a	88.85±12.27 b	69.50±0.05 a	0.94±0.06 b
		MH63	22.93±0.44 b	58.80±6.58 b	71.21±0.08 a	0.70±0.05 b
		CK	25.81±0.55 a	156.98±11.81 a	85.07±0.02 a	1.89±0.45 a
	M	T1c-19	23.14±0.64 b	56.83±8.81 b	65.15±0.06 a	1.81±0.47 b
		MH63	22.98±1.05 b	67.30±9.45 b	73.46±0.03 a	1.79±0.37 b
		CK	26.89±0.61 a	181.20±17.47 a	79.76±0.07 a	5.21±0.49 a
	H	T1c-19	23.22±0.56 a	52.45±4.96 b	63.42±0.02 a	4.50±0.63 b
		MH63	23.13±0.22 a	63.33±1.38 b	74.64±0.03 a	4.16±1.01 b
		CK	24.09±0.98 a	126.00±17.47 a	80.95±0.06 a	9.89±2.51 a
15th June	L	T1c-19	20.68±1.19 b	80.05±21.93 b	81.83±0.04 a	0.71±0.10 b
		MH63	21.05±1.09 ab	67.05±8.89 b	81.71±0.02 a	0.84±0.23 b
		CK	23.78±1.07 a	143.58±24.13 a	86.63±0.04 a	1.15±0.34 a
	M	T1c-19	21.16±0.87 b	63.43±11.88 b	79.51±0.02 a	1.24±0.55 b
		MH63	19.68±0.69 b	53.65±3.21 b	79.09±0.01 a	1.38±0.11 b
		CK	25.07±0.61 a	155.65±18.31 a	84.12±0.01 a	3.17±0.66 a
	H	T1c-19	21.36±0.61 b	59.60±4.68 b	79.29±0.02 b	3.90±1.03 b
		MH63	21.88±0.93 b	66.55±7.62 b	80.52±0.01 ab	4.09±0.11 b
		CK	24.22±0.43 a	127.38±9.36 a	87.96±0.03 a	5.97±0.99 a
25th July	L	T1c-19	17.24±0.67 a	19.85±2.22 b	44.45±0.04 b	0.39±0.48 b
		MH63	15.14±1.39 a	13.82±4.38 b	41.13±0.07 b	0.49±0.12 b
		CK	19.00±0.94 a	87.33±14.19 a	87.06±0.01 a	1.28±0.26 a
	M	T1c-19	16.04±2.27 a	17.53±5.39 b	41.49±0.04 b	0.57±0.18 b
		MH63	15.78±1.00 a	17.08±1.81 b	49.71±0.09 b	0.54±0.13 b
		CK	19.33±0.87 a	91.40±12.71 a	85.05±0.02 a	2.95±0.36 a
	H	T1c-19	16.71±0.83 a	19.18±2.16 b	49.68±0.06 b	1.20±0.09 b
		MH63	18.35±1.47 a	30.65±5.17 b	60.68±0.04 b	0.89±0.35 b
		CK	20.05±0.99 a	102.81±16.40 a	83.92±0.02 a	3.81±1.30 a

SD, sowing date; SR, sowing rate; RL, rice line; PL, panicle length; FG, filled grain number of main panicles; SS, seed set; Y, yield.

Table 4 Reproductive variables of three rice lines in transplanted field

Rice line	Panicle length (cm)	Filled grain number of main panicle (n)	Seed set (%)	Yield m^{-2} (kg m^{-2})
T1c-19	25.12±0.49 b	108.73±3.72 b	78.27±0.02 c	0.54±0.05 b
MH63	25.49±0.25 b	131.54±9.74 b	83.38±0.02 b	0.49±0.09 b
CK	27.69±0.11 a	246.10±8.94 a	92.55±0.01 a	0.63±0.02 a

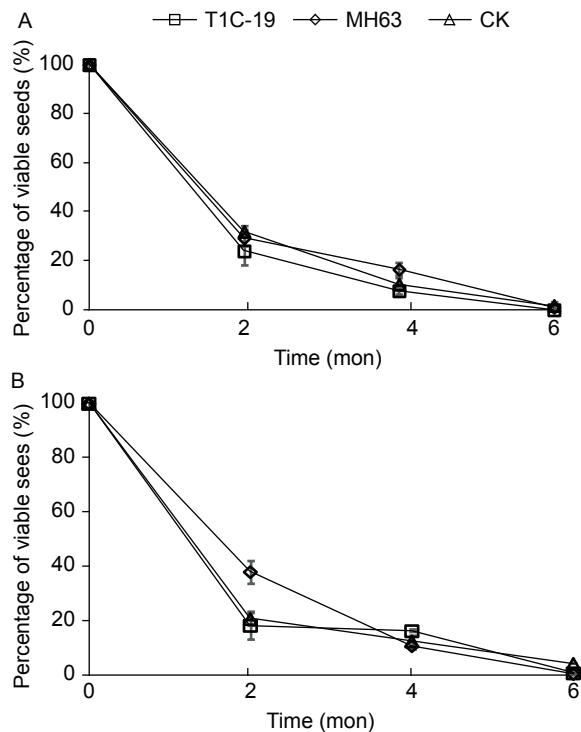


Fig. 5 Seed viability of the three rice lines after burial at 3 cm (A) and 20 cm (B).

3. Discussion

In the assessment of the competitive ability of transgenic plants, all stages in the plant life history should be considered, including emergence, seedling establishment, vegetative growth, and reproductive phase (fecundity and seed viability) (Parker and Kareiva 1996; Roberts *et al.* 2014).

Seedling emergence and establishment is the first developmental stage determining the potential weediness of a transgenic crop. Environmental factors associated with depth of seed in the soil and soil characteristics themselves as well as seed viability and vigor determine seedling emergence and establishment (Azhiri-sigari *et al.* 2005; Martinkova and Honek 2011; Rajala *et al.* 2011). Under our experimental conditions, environmental factors including soil type and its water content were consistent at each sowing rate in the same planting stage. Therefore, the similarity in seedling establishment between transgenic rice T1c-19 and the receptor rice MH63 indicated that the insertion of two transgenes did not induce any change of the intrinsic germination rate of MH63. Additionally, both the transgenic and the receptor lines had a similar seedling establishment rate to that of control.

The lowest seedling establishment rates for three rice lines were observed in the second stage, probably as a consequence of continuous rain-fall during the establish-

ment period. Waterlogging caused similar seed mortality among the three rice lines. Indirectly, we can also conclude that transgene did not enhance waterlogging tolerance for seedling establishment. Furthermore waterlogging was also responsible for the lowest weed occurrence in the second stage at 30 DAS.

Plant height is an important trait determining the competitiveness of plants (Caton *et al.* 1999, 2003; Cousens *et al.* 2003a, b; Mason *et al.* 2007; West *et al.* 2010). Tall cultivars are usually more competitive than short cultivars, because of better light interception that is directly associated with increased photosynthetic activity (Cudney *et al.* 1991). In our experiments, T1c-19 was as tall as or shorter than its receptor MH63 at all ages evaluated at three sowing rates at four stages. In most cases, both transgenic and the receptor rice lines were shorter than the control, thus it is unlikely that transgenic rice T1c-19 would display an increased competitive advantage related to height.

Tillering in rice is an important agronomic trait determining grain yield (Li *et al.* 2003). Differences of competitive ability are also positively associated to tillering capacity (Fang *et al.* 2011). In the wet direct-seeded field, at three sowing rates of four planting stages, tiller number of the three rice lines decreased as plants developed, with some lacking tillers at maturity. This age-related tiller mortality is most likely associated with density dependent relationships especially increased weed competition as plants mature. At any particular sowing rate, tiller number was similar among the three rice lines. Thus stacked transgenic rice T1c-19 performed similarly in relation to tillering to the receptor and control under weed competition. In the transplanted field; however, both the transgenic and receptor rice lines developed more tillers than the control. This implies that the three rice lines included in this study may tiller differentially depending on the planting systems, suggesting that in some cases, T1c-19 may have an increased potential for weediness by producing more tillers.

Flowering is the first step for seed setting. T1c-19 and its receptor rice MH63 flowered simultaneously at the first three planting stages but failed to flower at the fourth planting stage in both the wet direct-seeded and transplanted fields. Thus transfer of both of *cry1C** and *bar* genes did not affect flowering time of MH63. Days from sowing to the beginning flowering of most cultivars both in different transplanting and direct seeding timing in Jiangsu Province, China, vary between 70–125 days as reported by Dong *et al.* (2011) and Du *et al.* (2012). The flowering stage of T1c-19 will overlap with that of some rice cultivars in Jiangsu Province at varied degree. Resistance-gene flow from T1c-19 in hybrid seed production to the other cultivated rice planted in Jiangsu Province is inevitable where these plants occur in sympatry. Therefore, establishing appropriate isolation distances is

necessary for minimizing the risk of gene flow from T1c-19 to non-transgenic rice in adjacent fields.

The reproductive ability of plants is another determinant trait for weediness. T1c-19 and MH63 were similar in panicle length, filled grain number per main panicles, seed set, and yield m^{-2} at the same sowing rate at three stages of wet direct-seeded and transplanted fields. Compared to the control, reproductive variables of these two rice lines were usually significantly lower. Therefore transgenes *cry1C** and *bar* inserted into MH63 did not change its reproductive ability, which additionally was lower than that of the control.

Besides reproductive ability, other characteristics contribute to the adaptation and persistence of weeds (Baker 1974). Shattering is an adaptive trait for seed dispersal in wild plants (Zhou et al. 2012). If transgenic rice has higher seed shattering, it may have a better opportunity to survive in the field. Generally, *indica* cultivars exhibit relatively strong seed shattering, whereas some *japonica* cultivars do not exhibit it at all (Konishi et al. 2006). Therefore seed shattering should be an important trait in the assessment of transgenic rice, especially for *indica* cultivars. In the present study, seed shattering of T1c-19 was similar to that of its receptor MH63, but significantly lower than that of the control rice material. This may be unfavorable for T1c-19 to escape from harvest and persist in the paddy field. However, it is unpredictable what would be the shattering rate of the transgenic hybrid cultivar bred with T1c-19 as cytoplasmic male sterile (CMS) restorer. Additionally, combine harvesting, always causing quantitative losses in rice production (Alizadeh and Allameh 2013), is commonly practiced in both Jiangsu Province and other areas. Therefore possible dissemination potential of T1c-19 and hybrids bred with T1c-19 should be paid a close attention. Wild or weedy species developed dormancy mechanisms during evolution to enhance their survival under adverse natural or in human-disturbed environments by selection for an optimum time to germinate (Gu et al. 2008). Seed dormancy of the three rice lines was indirectly examined, and it was found that the three rice lines had similar weak dormancy. But no volunteers were observed in the field in the following year. This implied that seed survival capacity of the three rice lines under natural field environments was low.

Fitness is determined by a number of genetically controlled traits but it is also influenced by the environment (Guèritaine et al. 2002; Mercer et al. 2007; Yang et al. 2011, 2012; Hovick et al. 2012). Previous work showed that selection pressure affect plant fitness (Londo et al. 2010; Xia et al. 2011; Yang et al. 2011, 2012). Under high insect selection pressure, insect-resistant plants performed better on yield, seed set, and exhibited fitness benefits (Xia et al. 2011; Yang et al. 2011, 2012). Insect-resistant transgenic rice lines yielded less under low insect pressure (Kim 2008;

Xia 2010). Herbicide selection also affected reproductive fitness for non-transgenic and transgenic genotypes (Londo et al. 2010). Therefore, prior to commercialization of T1c-19, field assessments should be conducted to test the potential benefit and cost of transgenes under different insect and glufosinate pressures. Additionally, both transgenes of T1c-19 may escape to weedy rice if T1c-19 is released in field. If this happened, weedy rice with both transgenes could have a fitness advantage under herbicide (release from competition by normal, susceptible weedy rice) and insect pressure. Therefore, gene flow should be prevented if transgenic rice is commercially released.

Besides selection pressure, competition also impacts fitness (Rose et al. 2009; Hovick et al. 2012). The persistence of transgenic individuals in a population depends on their relative fitness and competitive ability against non-transgenic neighbors (Liu et al. 2015). Therefore, the relative competitive ability of resistant transgenic to non-transgenic rice with different proportions in mixed stands should be studied.

Characteristics of hybrid rice progeny often strongly segregate owing to the great genetic diversity of parental lines (Yuan 2002). Hybrid rice progeny that escapes harvesting may segregate into distinct biotypes through gene recombination, some of which might evolve into weedy rice. Interestingly, cytoplasmic-genetic male sterility gene provides direct evidence for some hybrid rice recently evolving into weedy rice (Zhang et al. 2015). Although no volunteer seedling of T1c-19 was found during the experimental period, hybrids bred with T1c-19 should be monitored closely in case of evolving into weedy rice with both insect and glufosinate resistant genes.

4. Conclusion

In summary, in the wet direct-seeded and transplanted rice fields, stacked (insect and herbicide-resistant) transgenic rice T1c-19 and its receptor MH63 were similar in vegetative growth and reproductive ability, but significantly lower than the control commercial hybrid rice cultivar. The stacked traits involved in this experiment were not expected to increase plant fitness under field condition without the relevant selection pressure. T1c-19 does not increase potential weediness and poses low risk under natural field conditions in middle of Jiangsu Province, China.

5. Materials and methods

5.1. Plant materials

The transgenic rice line T1c-19 contains two tightly linked single-copy genes, the insect-resistant gene *cry1C** under the control of the *Ubi* promoter and the herbicide-tolerant

bar gene under the control of the CaMV 35S promoter. It was obtained by *Agrobacterium*-mediated co-transformation of *indica* cultivar Minghui 63 (MH63), a restorer line used in several widely grown hybrids (Tang *et al.* 2006).

The *bar* gene conferring herbicide resistance is widely used as a selectable marker for transformation. MH63, the receptor conventional counterpart rice of T1c-19, is an elite *indica* CMS restorer line, extensively used in China in hybrid-rice breeding. Fengliangyou Xiang 1 (Hefei Fengle Seed Co., Ltd., Anhui, China) is a two-line *indica* hybrid (obtained by crossing Guangzhan 635 and Fengxianghui 1, herein also referred to as control) that is widely cultivated in Nanjing, China (Zhou *et al.* 2010).

5.2. Field trials

Field trials were conducted at the Jiangpu Experimental Station (32.011569°N, 118.624535°E), Weed Research Laboratory, Nanjing Agricultural University in Jiangsu, China. The experimental fields, where the studies were conducted from May 2013 to June 2014, were authorized by the Ministry of Agriculture of China. At the location, the experimental area was isolated by a 100-m wide corn crop, and was rotated by rice and wheat for 5 years.

Direct seeding The experimental field was divided into four sections (144 m² each), separated from each other by 60 cm. The experiments were conducted in the first, second, third and fourth sections on 25th May, 15th June, 5th July and 25th July (herein the first, second, third and fourth stages and referred to as I, II, III and IV, respectively). Each of the four sections was further divided into four sub-sections (36 m² each), representing four replications. Each sub-section was additionally divided into nine experimental plots (4 m² each). In each plot, the three rice lines were randomly planted at three sowing rates: 75 (low), 150 (medium), or 300 (high) seeds m⁻².

Transplanting Seeds of three experimental rice materials were sown in a separate rice seedling field on 25th May. This experiment was established in a complete randomized block design with four replications. Single seedlings were transplanted 20 cm apart from each other in the adjacent paddy field 30 days after sowing. Each replication was 18 m² and included three plots for three experimental rice lines. Each plot was 2 m×3 m, contained 176 plants in 17 rows. Replications were separated by 40 cm.

The cultivation practices used during growing period, including irrigation and fertilization were those commonly used in commercial rice production. However no action was taken for weed and any other pest control.

Measurement of variables in field Weed occurrence was investigated at 30 and 50 day after sowing in both the direct

seeded and transplanted fields. The number and species of weeds presented in each plot were recorded and their planimetric area in each plot was determined in five 0.1 m² samplings sites randomly selected. A relative coverage ratio was calculated as total canopy area of weeds in each plot divided by plot area (4 m²).

At 10 day after sowing, the number of the established seedlings was recorded and seedling establishment rates were calculated. For the measurement of other variables, the tallest ten individual plants were selected in each plot. All variables were measured according to the standard evaluation system for rice available from the International Rice Research Institute (IRRI) (2002). These variables included four vegetative traits (seedling establishment rate, plant height, tiller numbers per plant and above-ground dry biomass) and four reproductive ones (panicle length, full grain number per panicle, seed set, and yield). Plant height and tiller number were measured at 30, 50, 70 day after sowing and maturity.

Pollen viability was evaluated at flowering. Pollen grains were stained with 1% iodine potassium iodide (I₂/KI) solution, which is widely used for staining starch and the starch content in pollen grains, and serves as an indicator of viability (Jiang and Ramachandran 2011). The other traits were measured at maturity. Seed shattering was evaluated according to Song *et al.* (2011).

Monitoring of volunteer seedlings After the selected 10 plants were harvested for testing vegetative and reproductive traits, the rest of the plants in each plot were left in the field for monitoring volunteer seedlings. The volunteer seedlings were counted every 20 days in two periods: from rice harvested to the end of 2013 and from May to July of 2014.

Seed survival over winter in the field Twenty seeds of each rice line were individually placed into small nylon mesh bags (0.425 mm sieve) and sealed tightly. Then one small bag of each line was put in a large nylon bag of the same mesh and closed tightly. The large nylon mesh bags were buried at 3 cm depth and 20 cm depth in the experimental field (snowy and rainy conditions, but no irrigation) in November 2013 in a completely randomized design with four replications. From December 2013 to June 2014, four large bags were retrieved randomly at 2, 4 and 6 months after burial. The germinated seeds in each small bag were counted after 2 weeks. The seeds remaining intact were subjected to a viability test using triphenyl tetrazolium chloride. The germination percentage of each type of seed was calculated:

$$\text{Seed survival} = (\text{Number of germinated seeds} + \text{Number of stained seeds}) / \text{Total number of seeds}$$

During the experiment, the average highest and lowest temperature in the first, second and third month were approximately 8.1–11.2°C and 0.5–1.4°C, respectively. From

the fourth to sixth month, temperature elevated to average the highest and lowest temperature of 16.6–27.6°C, and 7–17.7°C, respectively.

5.3. Data analysis

Statistical analysis was carried out by conducting analysis of variance (ANOVA). Means were separated using Duncan's multiple range test ($P < 0.05$), except for means of reproductive variables of three rice lines in transplanting field, which were separated using least significant difference (LSD) test ($P < 0.05$). All the statistical analyses were carried out using the SPSS (18.0, SPSS Inc., Chicago, IL, USA) software package. Data of plant height of the same rice line at the same sowing rate from the first to the fourth sowing stage were pooled for analysis due to similar results in the different sowing stages.

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